



Europäisches Patentamt

European Patent Office

Office européen des brevets

Publication number:

**0 154 734
B1**

⑫

EUROPEAN PATENT SPECIFICATION

④ Date of publication of patent specification: 29.08.90

⑪ Int. Cl.⁵: **G 01 N 33/533,
G 01 N 33/534**

⑦ Application number: 84301769.0

⑫ Date of filing: 15.03.84

⑤ Immediate ligand detection assay, a test kit and its formation.⑥ Date of publication of application:
18.09.85 Bulletin 85/38④ Publication of the grant of the patent:
29.08.90 Bulletin 90/35④ Designated Contracting States:
AT BE CH DE FR GB IT LI NL SE⑤ References cited:
EP-A-0 070 686
GB-A-2 095 830
US-A-4 000 252
US-A-4 259 313
US-A-4 271 139
US-A-4 382 074⑦ Proprietor: **IMMUNEX CORPORATION**
51 University Building Suite 600
Seattle Washington 98101 (US)⑦ Inventor: **Bertoglio-Matte, Jacques Henri**
4849 Terrace Drive N.E.
Seattle Washington 98105 (US)⑦ Representative: **Sheard, Andrew Gregory et al**
Kilburn & Strobe 30, John Street
London WC1N 2DD (GB)**EP 0 154 734 B1**

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European patent convention).

Courier Press, Leamington Spa, England.

EP 0 154 734 B1**Description**

The present invention relates to an assay for detecting the presence of small amounts of organic material, and more particularly to an assay to detect the presence of organic materials by monitoring the light energy produced when radioactive organic molecules of interest biochemically and specifically bind to a binding structure.

In clinical applications, in research and in industry, a need exists to detect the presence of minute amounts of organic material, such as antigens, antibodies, hormones, metabolites, enzymes, and drugs. In clinical situations, detecting the presence or absence of metabolites, hormones, or other organic factors in serum or in other body fluids may be useful in the diagnosis of many clinical conditions, such as pregnancy, infection, blood disorders, hepatitis, etc. The detection and quantitative analysis of organic agents is often required in immunological, biological, chemical, or other types of scientific and medical research. In industry, assays for organic materials are utilized in quality control procedures for the production of chemicals and in monitoring the pollution of water.

Of the numerous chemical and biological assays that have been developed to detect organic materials, of relevance to the present invention are precipitation and agglutination assays. In a typical precipitation assay, the organic material interacts with a reactant to form a complex that falls out of solution. In agglutination reactions, the organic substance of interest cross-links an insoluble reactant to cause the reactant to flocculate. Optical scattering techniques are commonly used to measure the flocculation. A drawback of precipitation and agglutination assays is that they are not as sensitive as radioimmunoassays. Also, although optical techniques have been developed to improve the sensitivity of these assays, these techniques require specialized equipment and analysis.

Another type of known assay for organic materials involves labeling either the organic material or a reactant thereto with a radioactive, fluorescent or other type of tracer substance to ascertain the extent to which the organic material has coupled with its reactant.

Radioimmunoassay is one of the most common types of these "tracer" assays. Radioimmunoassay involves combining a known amount of radiolabeled organic material with a sample containing an unknown amount of unlabeled organic material of interest together with a specific antibody that binds indiscriminantly to the labeled and unlabeled organic materials to form a complex. After an incubation period, the unbound organic materials are separated from the bound organic materials, typically by precipitation of the complex with polyethylene glycol, adsorption of the unbound material with activated charcoal or utilization of solid-phase reagents. Then the radioactivity of either of these two fractions is measured. A certain amount of the labeled and unlabeled organic material will be bound to the reactant, with the amount of the bound labeled organic material being inversely related to the quantity of unlabeled inorganic material present in the sample being tested.

A drawback of the radioimmunoassay is that the procedures for separating the bound organic materials from the unbound require a significant number of time-consuming operations that are often complicated and expensive. The separation procedures involve repeatedly washing the complex of organic material and antibody with a rinsing solution and/or centrifuging the mixture to remove the unbound organic material from the reactant, thereby generating radioactive waste material with each washing. By the time that the separation process has been completed, significant volumes of radioactive waste material are produced. This waste material is not only expensive to dispose of, but also presents a potential health hazard to persons handling the material, including during the separation procedures.

In an assay utilizing fluorescence, the organic material may be labeled with an appropriate fluorescer, such as fluorescein isothiocyanate. The extent to which the fluorescer labeled organic materials bound to a specific reactant can be examined under a light microscope with a suitable light source and filters to provide incident light of the proper wavelength to cause fluorescence.

An example of a particular fluorescence technique is disclosed in U.S. Patent 4,161,515 wherein an unknown organic compound, whose presence is being investigated, is mixed with: 1) a known quantity of antibody against the organic compound; 2) an organic analog, having a fluorescer bound thereto, which competes with the unknown organic compound for the antibody; and 3) an antibody for the fluorescer. The competition between the unknown organic material and the known analog-fluorescer effectively reduces the concentration of the antibody, thereby causing more of the fluorescer-antibody to combine with the analog-fluorescer. This, in turn, causes a corresponding change in the emission spectrum of the fluorescer.

A tracer assay that utilizes both fluorescence and radioactive substances is disclosed by U.S. Patent 4,000,252 wherein a known quantity of radiolabeled antigen and a sample containing an unknown amount of unlabeled antigen are placed within an immunoscintillation cell. An insolubilized or solid phosphor, which is chemically or physically associated with an antibody to the antigens, is also added to the cell. The unbound antigens are washed from the cell and then the luminescence emitted by the phosphor due to activation from the radioactive energy from the bound labeled antigens is measured inside a scintillation counter. Removal of the unbound antibodies from the cell requires several washing procedures that are not only time consuming, but also produce significant quantities of radioactive waste material.

Assays that combine tracer techniques with agglutination are disclosed by U.S. Patents 4,108,972 and 4,271,139. In the '972 patent, microscopic carrier particles, each containing a fluorescent tracer material, and a biological reactant to the antibody or antigen being investigated, are placed in suspension. When the

EP 0 154 734 B1

antigen or antibody is added to the suspension, it binds to the reactant coating to cause flocculation of the carrier particles. The flocculated material is then separated from the suspension fluid and other constituents by numerous washings. Thereafter, the flocculated material is dissolved and then assayed by fluorescence techniques to determine the quantity of organic material present.

5 In the '139 patent, tritiated (radioactive) latex particles coated with antigen and polystyrene scintillant particles coated with the same antigen were placed in an aqueous medium with a sample containing an unknown quantity of a corresponding antibody. The number of tritiated antigen coated latex particles linked to the antigen coated scintillant particles is related to the concentration of antibody present. Also, when the two particles are linked together by the antibody, the radioactive energy from the tritiated
10 particles initiates scintillation within the scintillant particles. Scintillations are then measured by an appropriate detector, with the detected level of scintillation being indicative of the quantity of the antibody present. Addition of an unknown quantity of non-radioactive antigen then competes with the antigen coated bead binding to the antibody, thereby reducing bead agglutination, scintillation and signal. A drawback of this particular assay is that it requires both tritiated latex particles and polystyrene scintillant
15 particles to be coated with antigen, which increases the expense and complexity of the assay. In addition, relative to the standard radioimmunoassay discussed above, extremely large suspension volumes are required for the assay to operate properly. The assay process of the '139 patent also requires the availability of relatively pure antigen to be used to bind to the two types of carrier particles. Relatively pure samples of antigen are both expensive and difficult to obtain.

20 US-A-4382074 is a continuation-in-part of US-A-4271139 and describes a similar agglutination assay with an alternative embodiment of adding free antigen to the sample to compete with the antigen-coated particles for the antibody to be assayed.

US-A-4259313 discloses polymeric latices which have fluorescent rare earth chelates incorporated into the latex beads. The beads are added to a solution of the rare earth chelate in a water-miscible solvent,
25 and then water added to the latex to transfer the hydrophobic fluorescer into the beads.

Thus, it is a principal object of the present invention to provide an accurate, inexpensive, and rapid assay procedure and test kit for detecting the presence of extremely small amounts of organic materials.

A particular object of the present invention is to provide an assay of equivalent accuracy to present techniques, but which does not require highly skilled personnel or large amounts of time to perform using
30 standard commercially available equipment.

A further particular, but highly important object of the present invention, is to provide an assay procedure that produces only a minimum volume of radioactive waste and requires only a minimum amount of handling of hazardous substances.

An additional particular object of the present invention is to provide an assay that can be used to
35 rapidly test a large number of samples.

Another particular object of the present invention is to provide an assay that utilizes water as a suspension medium.

Therefore, according to a first aspect of the present invention there is provided a process for the detection of a radiolabelled reactant comprising:

40 a) placing in an aqueous suspension a plurality of support particles impregnated with a fluorescer and to which ligands have been attached;

b) adding to the suspension fluid a radiolabelled reactant capable of specifically biochemically binding to the ligand, said radiolabelled reactant emitting radiation energy capable of activating the fluorescer upon the binding of the reactant to the ligand, the radiolabelled reactant then being disposed in close
45 enough proximity to the support particles to cause the radiation energy from the radiolabelled reactant to activate the fluorescer to produce light energy, whereas any radiolabelled reactant that does not bind to the ligand is generally too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and

50 c) measuring the light energy emitted by the fluorescer with the entire quantities of the support particles, bound and unbound radiolabelled reactant together in aqueous suspension.

A second aspect of the invention relates to a competitive reactant assay process, comprising:

a) combining together in an aqueous medium:

a sample to be assayed containing an unknown amount of cold ligand reactant;

a known quantity of radiolabelled ligand reactant; and

55 a plurality of support particles having fluorescer integrated therewith capable of emitting photons when activated by the radiation energy emitted by the radiolabelled ligand reactant and having ligand attached to the outer surface thereof, said ligand capable of indiscriminately binding with both said cold and radiolabelled ligand reactant whereupon the binding of the ligand with the radiolabelled ligand reactant positions the radiolabelled ligand reactant close enough to the support particles to activate the
60 fluorescer to emit photons through the aqueous medium, whereas the unbound radiolabelled ligand reactant is generally positioned too far away from the support particles to enable the radioactive energy emitted thereby to activate the fluorescer; and

b) measuring the photons emitted by the fluorescer with the entire quantities of cold ligand reactant containing sample, bound and unbound radiolabelled ligand reactant and support particles combined
65 together in the aqueous medium.

EP 0 154 734 B1

A third aspect of the present invention relates to a test kit for the detection of a ligand reactant that biochemically and specifically binds to a ligand in an aqueous medium with all of the components of the test kit together in the aqueous medium, said test kit comprising:

- a) support particles, preferably in a predetermined quantity, having a fluorescer incorporated therewith and the ligand attached thereto; and
- b) a radiolabelled reactant, preferably in a predetermined quantity, having substantially the same specificity for the ligand as the ligand reactant; said support particles being such that when said kit is in use any of said radiolabelled reactant bound to the ligand is positioned close enough to the fluorescer to activate the fluorescer by the radioactive energy transmitted through the aqueous medium from the radioactive reactant at a level corresponding to the proportion of the radiolabelled reactant to the ligand reactant present in the aqueous medium but if not bound to the ligand is too far away from the fluorescer to result in other than background levels of fluorescer activation.

The objects are achieved in accordance with the present invention by providing a test kit and assay procedure which produces light energy at a level related to the amount of organic material present in a sample being tested. The light energy is produced by a fluorescer which is integrated into support bodies in the form of beads or other structures. The support bodies are coated with a ligand that is capable of specifically binding to the organic material or reactant of interest. When the present invention is used as a direct assay, the reactant is radiolabelled. Then, the sample containing the radiolabelled reactant is mixed in an aqueous solution with the support bodies, causing the reactant to bind to the ligand. This places the radiolabelled reactant in close enough physical proximity to the support bodies to cause the radiation energy emitted from the radiolabelled reactant to activate the fluorescer integrated into the support bodies, thereby causing the fluorescer to emit light energy. The level of the light energy produced is related to the amount of reactant that is bound to the ligand, which in turn, is indicative of the amount of reactant present in the sample being tested.

The present invention may also be utilized as a competitive assay to determine the amount of a reactant contained in a sample. In this situation, a known amount of the reactant is radiolabeled. The reactant of interest in the sample being tested remains unlabeled. Both the labeled and unlabeled reactant are capable of specifically binding with the ligand. In the assay process, both the labeled and unlabeled organic materials are placed in an aqueous solution, together with the support bodies that have been impregnated with the fluorescer and coated with the ligand. Since the ligand does not favor either the labeled or unlabeled reactant, the reactants bind to the ligand in proportion to their relative amounts present in the aqueous solution. However, only the radiolabeled reactant that binds to the ligand is brought in close enough proximity to the support bodies to cause the radiation energy emitted thereby to activate the fluorescer integrated into the support bodies. Thus, the level of light energy produced by the fluorescer is inversely proportioned to the quantity of unlabeled reactant present in the sample.

In both the direct and competitive assays, radiation energy emitted by the radiolabeled reactant, which is capable of activating the fluorescer, has a limited range of travel in water. Thus, the reactant that has not bound to the ligand is too far away from the support structures to permit the radiation energy emitted therefrom to reach the fluorescer. Thus, since only the radiolabeled reactant that actually binds to the ligand is responsible for causing the fluorescer to emit light energy, the radioactive reactant that has not bound to the ligand need not be separated from the ligand-reactant complex prior to measuring the level of light energy emitted by the fluorescer. Thereby, as a consequence of the present invention, the laborious and time-consuming procedure of separating the unbound labeled reactant from the bound complexes by centrifuge, precipitation, washing and other procedures is eliminated, as are the large quantities of radioactive waste material produced by these separation techniques.

In a further aspect of the present invention, a unique technique is provided for integrating the fluorescer into the support bodies. Initially, the support bodies are soaked in a solvent for the fluorescer which is miscible in water to dehydrate the bodies. Thereafter, the bodies are placed in a solution composed of the fluorescer and solvent so that the fluorescer is integrated and/or adsorbed into the bodies. Then, the bodies are removed from the solvent and then placed in an aqueous solution which causes precipitation of the fluorescer within the bodies, thereby locking the fluorescer therein. By this technique, the fluorescer is integrated within the interior of the bodies so that the radiolabeled reactant is placed in very close proximity to the fluorescer upon binding to the ligand, which is disposed on the exterior of the bodies.

In accordance with the present invention, support bodies or particles in the form of beads or other structures are impregnated and/or coated with a material capable of fluorescence when excited by radioactive energy. The beads are coated with a ligand that is capable of specifically binding to a reactant of interest by covalently linking or directly attaching the ligand to the beads. The beads are then mixed in a water-based solution containing the reactant and has been radiolabeled. Upon binding of the radiolabeled reactant to the ligand, the fluorescer integrated into the beads is placed in close enough physical relationship to the reactant to allow the radiation energy emitted from the reactant to activate the fluorescer thereby causing the fluorescer to emit light energy. The level of light energy emitted, which is indicative of the extent to which the ligand is bound to its reactant, may be conveniently measured with a scintillation counter or other monitoring device employing a photomultiplier tube.

The radiation energy emitted by the radiolabeled reactant molecules has a very limited range of travel

EP 0 154 734 B1

in water. The reactant molecules that have not bound to the ligand are, for the most part, located too far away from the ligand to enable the radiation energy emitted from these unbound reactant molecules to reach the fluorescer in the support structure, i.e., beads. Since there is very little likelihood of chance excitation of the fluorescer by the radioactive energy of these unbound reactant molecules, the reactant molecules that have not bound to the ligand need not be separated from the ligand-reactant complexes prior to scintillation counting of the light energy emitted by the fluorescer excited by radioactive energy from the reactant molecules that have bound to the ligand. Thus, the traditional laborious and potentially hazardous procedure of separating the unbound reactant from the ligand-reactant complexes is eliminated.

The present invention may also be used in conjunction with a competitive assay procedure. In this instance, the support bodies, having a fluorescer integrated therein and coated with an appropriate ligand, are placed in an aqueous solution containing a known quantity of radiolabeled reactant and a sample containing an unknown amount of the same, but unlabeled reactant. Since the ligand does not favor binding to either the labeled or unlabeled reactant over the other, the amount of labeled reactant binding to the ligand will be inversely proportional to the quantity of unlabeled reactant present in the sample. Prior to the assaying of a particular sample, different known amounts of unlabeled reactant are mixed together in individual vials with constant amounts of radiolabeled reactant and with a fixed quantity of ligand coated beads. The level of fluorescent energy generated by excitation of the fluorescer from the radiolabeled reactant that has bound to the ligand is measured for each vial containing a known amount of the unlabeled reactant. From the results of these measurements, a standard curve may be prepared depicting the level of fluorescent energy measured per quantity of unlabeled reactant present. Then, when a particular sample containing an unknown amount of unlabeled reactant is assayed, the concentration of the unlabeled reactant in the sample may be determined from the standard curve once the level of fluorescent energy being emitted is measured.

As in the direct assay procedure described above, in the competitive assay, only the radiolabeled reactant molecules that are bound to the ligand are in close enough proximity to the beads to allow the radiation energy emitted by the labeled reactant to bombard the fluorescer integrated into the beads. The detected level of fluorescent energy, therefore, is a reflection of the proportion of the radiolabeled reactant which actually binds to the ligand. As a consequence, there is no need to wash the beads or otherwise attempt to remove the unbound radioactive reactant from the ligand; instead, the level of fluorescent energy may be measured with all of the components of the assay still present in the vial. Moreover, the time required to complete the assay is limited only by the ligand-reactant binding reaction rate of the system under investigation.

As noted above, the ligand is bonded to and the fluorescer is integrated with a structural support, such as beads. Various types of beads may be utilized, such as polyacrylamide, acrylamide, agarose, polystyrene, polypropylene, polycarbonate or Sepharose® 4B beads (from Pharmacia Fine Chemicals, Uppsala, Sweden). The present invention also may be carried out with other shapes or types of support structures, for instance latex particles, as long as the ligand molecules can be covalently or otherwise attached thereto and a fluorescer integrated therewith.

Beads, such as the Sepharose® 4B beads noted above, are commercially available in an activated state. Compounds, such as cyanogen bromide, are incorporated in the beads to covalently bind with certain ligands. The process by which the ligand is bound to the beads is dependent on the type of bead and the particular ligand employed. For instance, for Sepharose® 4B beads activated with cyanogen-bromide, a ligand in the form of Staphylococcus aureus protein A or an antibody to a specific reactant may be bound to the beads by placing the beads in a solution containing the protein A or antibody and an appropriate buffer. Thereafter, the excess protein A or antibody is washed away and the remaining active sites on the beads to which no protein A or antibody had attached are blocked with an appropriate blocking agent, such as glycine. This prevents the reactant of interest and others from binding directly to the beads, rather than to the ligand. Other techniques for bonding a ligand to beads include the use of carbodiimide coupler, tannic acid, glutaraldehyde and polyethylene glycol.

As noted above, in accordance with the present invention, a fluorescer is integrated within the beads to give off light energy when radiolabeled reactant is brought in close enough proximity to the fluorescer to cause excitation thereof, i.e., by binding to the ligand on the bead surface. Various types of fluorosceners may be used; however, since the process of the present invention takes place in an aqueous solution, the fluorescer must be insoluble in water so that it does not dissociate from the beads during the assay procedure. Also, the fluorescer employed must be excitable to a higher energy state by the particular wavelength of the radioactive energy rays emitted by the radiolabeled reactant, and also must release sufficient light energy when returning to its normal energy state to be detected by a scintillation counter or other detection device utilizing a photomultiplier tube. An example of a fluorescer that has been found to meet these requirements for use with radioactive energy in the form of beta rays or auger electrons is diphenyloxazole (hereinafter "PPO").

The present invention involves a novel process for integrating a fluorescer into a support structure, such as beads. Since the fluorescer is insoluble in water, an appropriate transfer medium, in which the fluorescer is soluble, must be used to incorporate the fluorescer into the beads. Moreover, the transfer medium itself must be miscible with water.

The novel method of the present invention for integrating the fluorescer into the beads includes

EP 0 154 734 B1

soaking the beads in an appropriate transfer solvent for the fluorescer that is miscible with water thereby to dehydrate the beads. Thereafter, the beads are incubated in a solution of the fluorescer and solvent. The fluorescer, which is in solution, is absorbed into the bead. The excess solvent is then discarded and the fluorescer is precipitated, adsorbed and/or integrated inside the bead by adding water or a buffered saline solution. Precipitating the fluorescer within the beads locks the fluorescer therein. Next, the beads are washed to remove the excess precipitated fluorescer and then resuspended in a solution containing both a detergent, such as Tween® 20, to prevent the beads from sticking together and gelatin to bind to any sites on the surface of the beads that either have not been blocked by the previously employed blocking solution or bound to a ligand. This lowers the nonspecific binding tendency of the beads. Finally, a bactericide, such as sodium azide, can be applied to the beads to prevent the growth of bacteria thereon.

Applicant has found that dimethyl sulfoxide (hereinafter "DMSO") may be utilized as a transferring solvent if PPO is used as a fluor. PPO is soluble in DMSO, and DMSO is miscible in water. Also, the DMSO does not hinder the ability of the PPO to precipitate within the beads when subjected to an aqueous solution.

The process of the present invention requires the use of radiolabeled reactants. The radiolabeled reactants are biologically and chemically identical to an unlabeled reactant, with the exception that the labeled reactants emit radioactive energy due to the decaying of the radioactive isotope present.

The technique used for labeling of the reactant varies with the type of radioactive isotope employed. For instance, labeling can be accomplished by replacing one of the atoms of the reactant molecules with a corresponding radioactive isotope. A hydrogen atom could be replaced with: tritium, ³H; a carbon atom replaced with carbon-14, ¹⁴C; or a strontium atom replaced with strontium-38, ³⁸Sr. In another labeling process, rather than replacing the atoms of the reactant with a radioactive isotope, an isotope may be added to the reactant molecule. Such radioactive isotopes in common use include: iodine-125, ¹²⁵I; and iron-59, ⁵⁹Fe. In situations in which biological organisms or parts of those organisms are capable of synthesizing proteins, labeling can be carried out by culturing the organism with an appropriate radiolabeled precursor, such as methionine-35 (³⁵S), to cause the organism to incorporate the isotope into its products. Many reactants, such as antigens, antibodies, hormones, hormone receptors, enzymes, or enzyme cofactors, are readily available in radiolabeled form from various commercial sources.

Radioactive isotopes used to label the reactant have only a limited range in water so that, for the most part, only the radioactive energy from the labeled reactant that binds to the ligand actually activates the fluorescer. If PPO is used as a fluorescer, applicant has found that isotopes that emit either beta rays or auger electrons from gamma ray emissions fulfill this requirement. PPO does not fluoresce from the gamma rays themselves which have a longer range of travel than beta rays or auger electrons.

The assay process of the present invention may be utilized in conjunction with any ligand-reactant combination or system that specifically binds together and in which the reactant may be radiolabeled without affecting its specificity for the ligand. Examples of such ligand-reactant combinations include antibodies and their corresponding antigens. Either the antibody or antigen may be attached to the bead or other type of support structure to function as the ligand, with the corresponding antigen or antibody serving as the reactant. Another ligand-reactant system may be composed of protein A and corresponding immunoglobulins. The need to ascertain the presence of antigens, antibodies, and immunoglobulins exists in many clinical and research settings, especially in the detection of diseases and allergies and in investigations of the immune system.

Additional ligand-reactant systems with which the present invention is especially useful include: 1) lectins-glycoproteins; 2) biotin-avidin; 3) hormone receptor-hormone; 4) enzyme-substrate or cofactor; 5) RNA-DNA; and 6) DNA-DNA. It is to be understood that in the present invention either element may serve as the ligand or reactant.

The present invention also may be of particular value in conducting enzyme kinetic studies. The enzyme may serve as a ligand to bind with the radiolabeled reactant. Since all of the reagents of the assay system are always present together in the same vial and because the reagents are suspended in an aqueous-based buffer rather than an organic solvent, kinetic experiments may be conveniently carried out by simply measuring the light energy emitted from the same vial at different time intervals to determine the reaction rate of the reagents. Also, in the RNA-DNA system, the commonly used Northern, Southern and Western Blot tests may be advantageously replaced with the assay of the present invention.

Example I**Immunoglobulin-G Direct Detection Assay With Tritium Labeling**

To use the assay of the present invention to detect the presence of immunoglobulin-G, cyanogen-bromide activated Sepharose® 4B beads (obtained from Pharmacia Fine Chemicals, Uppsala, Sweden) are covalently coated with Staphylococcus aureus cowan strain 1 protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) or with anti-immunoglobulin-G antibody. This is accomplished by swelling and washing one gram of cyanogen-bromide activated Sepharose® 4B beads in one millimolar hydrochloric acid. Then, two milliliters of the washed beads are placed in a solution composed of either two milligrams of protein A or 15 milligrams of the immunoglobulin-G fraction of a rabbit antiserum to the human immunoglobulin-G heavy chain, together with sodium bicarbonate buffer, pH 8.3, containing 0.5 molar saline. The suspension is incubated for two hours at room temperature and then the excess protein is washed away by

EP 0 154 734 B1

centrifuging. Thereafter, the remaining active sites on the beads are blocked with 0.2 molar glycine. The beads are next washed with acetate buffer and bicarbonate buffer.

A fluorescer in the form of PPO is next incorporated into the beads. This is accomplished by dehydrating the coated beads by soaking them in DMSO for 15 minutes to remove any water in the beads that would cause premature precipitation of the PPO since PPO is insoluble in water. This step is repeated twice more and then the excess solvent removed by sedimentation. The beads are next incubated for 30 minutes at room temperature in a 20 to 40 percent weight by volume solution of PPO in DMSO. After incubation, the excess solution is discarded and the PPO precipitated inside the beads by adding ten volumes of either phosphate buffered saline (hereinafter "PBS") or water. The suspension is then washed five times in PBS to remove the excess precipitated PPO which is not bound inside the beads. Thereafter, the beads are resuspended at a final concentration of ten percent volume/volume in PBS supplemented with 0.5 percent volume/volume Tween 20 (Registered Trade Mark) as a detergent to help prevent the beads from sticking together and 0.1 percent weight/volume gelatin to bind to the remaining sites on the beads which were not blocked by reaction with protein A, immunoglobulin-G antibody or the glycine. This minimizes any nonspecific binding of radiolabeled reactant to the beads. Lastly, sodium azide, NaN_3 in an amount of 0.01 percent weight/volume, can be added to prevent bacterial growth.

In a direct assay for immunoglobulin-G, 50 microliters of the protein A or anti-immunoglobulin-G antibody coated beads are mixed together in scintillation vials with various concentrations of immunoglobulin-G labeled to a specific activity of 5×10^{12} counts per minute per millimole with ^3H -acetic anhydride. PBS supplemented with 0.5 percent volume/volume Tween[®] is added to the vials to bring the total volume in each vial up to a total of 3.0 milliliters. Then, the light energy produced by the bombardment of the PPO with the beta rays from the radiolabeled immunoglobulin-G is directly measured by a scintillation counter. The results of the assay wherein beads coated with protein A were utilized, shown in Table I, indicate that increasingly higher counts per minute were obtained by increasing the concentration of tritium-labeled immunoglobulin-G in the sample. PPO-impregnated Sepharose[®] 4B beads that were not coated with either a protein or anti-immunoglobulin-G antibody were used as a control, as also shown in Table I.

TABLE I**DIRECT DETECTION ASSAY FOR HUMAN IMMUNOGLOBULIN-G**

Bead Source	Tritium Labeled Human Immunoglobulin-G (Micrograms)	Counts Per Minute Determined After 5 Minute Incubation
50 Microliters of PPO Integrated Sepharose 4B Beads Coated with protein A	5 10 20 40	4221 5375 6140 7286
50 Microliters of PPO Integrated uncoated Sepharose 4B Beads	40	35

Example II**Immunoglobulin-G Direct Detection Assay With Iodine 125**

This Example is identical with Example I, except that the protein A or anti-immunoglobulin-G antibody coated beads were mixed with various concentrations of human immunoglobulin-G labeled with ^{125}I to a specific activity of 8×10^4 counts per minute per millimolar by the Chloramine T method. The protein A or anti-immunoglobulin-G antibody coated beads were placed in scintillation vials with various concentrations of ^{125}I labeled human Immunoglobulin-G and then the vials were placed directly into a scintillation counter to measure the resulting level of photon emission. The results of these tests are shown in Table II for 5-minute and 60-minute incubation periods. These data confirm that the assay can be measured immediately after mixing labeled reactant with PPO-impregnated ligand bound beads.

EP 0 154 734 B1

TABLE II

DIRECT DETECTION ASSAY FOR
HUMAN IMMUNOGLOBULIN-G

HUMAN IMMUNOGLOBULIN-G			
			Counts Per Minute Determined After
	<u>Bead Source</u>	<u>Iodine-125 Labeled Human Immunoglobulin-G</u>	<u>5 min/60 min Incubations</u>
10	75 Microliters of PPO	125 ng	10596/10238
	Integrated Sepharose	375 ng	14880/14910
	4B Beads Coated with	500 ng	18132/19638
15	protein A	750 ng	26216/27640
		1000 ng	33876/37784
		1500 ng	40066/41846
20	75 Microliters of PPO	125 ng	11030/11874
	Integrated Sepharose	375 ng	16762/18198
	4B Beads Coated with	500 ng	22684/24985
	anti-immunoglobulin-G	750 ng	24088/25946
	antibody	1000 ng	25904/31514
25		1500 ng	30056/37068

Example III

30 Competitive Assay for Immunoglobulin-G

Cyanogen-bromide activated Sepharose® 4B beads (Pharmacia Fine Chemicals, Uppsala, Sweden) are coated with either protein A (0.1 milligrams per milliliter of gel) or anti-immunoglobulin-G antibody (1.5 milligrams per milliliter of gel) in the manner set forth in Example I. Also, the Sepharose 4B beads are impregnated with the fluorescer PPO in the manner discussed in Example I. Fifty-microliter quantities of the prepared beads are mixed together in scintillation vials with 0.5 micrograms of ¹²⁵I-human immunoglobulin-G and increasing concentrations of unlabeled human immunoglobulin-G. The samples are then brought up to three milliliters each by adding PBS supplemented with 0.5 percent volume/volume of Tween®. The sample is then immediately counted for photon emission levels.

From the photon emission level measurements, standard inhibition curves were plotted in terms of the percent of inhibition of binding of the radiolabeled immunoglobulin-G caused by different quantities of unlabeled immunoglobulin-G. The inhibition curves using both anti-immunoglobulin-G antibody and protein A as ligands are shown in FIGURE 1. A sample containing an unknown amount of immunoglobulin-G can now be measured for percent of inhibition of the radiolabeled reactant and from this measurement the quantity of immunoglobulin-G present can be determined by using FIGURE 1.

Example IV

Thyroxin Determination

This Example involves the use of the present invention to develop a standard inhibition curve for the detection of thyroxin. Cyanogen-bromide activated Sepharose® 4B beads were coated with protein A and then impregnated with PPO in the manner described in Example I. Two hundred microliters of the prepared beads were pipetted into individual scintillation vials together with 400 microliters of a rabbit anti-thyroxin antiserum (obtained from Abbott Laboratories, Chicago, IL). Twenty-five microliters of various concentrations of unlabeled thyroxin (obtained from Abbott Laboratories, Chicago, IL) were added to each vial. Next, 100 microliters of thyroxin labeled with the isotope iodine-125 (obtained from Abbott Laboratories, Chicago, IL) were added to the vials. Lastly, the volume of the reaction mixture in each vial was brought up to three milliliters by the addition of PBS supplemented with 0.5 percent volume/volume of Tween®. The photon emission level in each vial was then measured in a scintillation counter and the results plotted in FIGURE 2 on a probability-log format. As shown in FIGURE 2, as the volume of unlabeled thyroxin added to the vials increased, the proportion of labeled thyroxin that bound to the beads decreased as expected. This standard inhibition curve can be used to determine the amount of thyroxin present in an unknown sample by using the same protocol described above in Example III to determine the percent of radiolabeled thyroxin that is inhibited from binding on the beads. From this value, the amount of thyroxin present in the sample may be conveniently read from the curve.

As will be apparent to those skilled in the art to which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit

EP 0 154 734 B1

or essential characteristics of the invention. The particular embodiments of the immediate ligand detection assay method, described above, are therefore to be considered in all respects as illustrative and not restrictive.

5 Claims

1. A process for the detection of a radiolabelled reactant comprising:
 - a) placing in an aqueous suspension a plurality of support particles impregnated with a fluorescer and to which ligands have been attached;
 - 10 b) adding to the suspension fluid a radiolabelled reactant capable of specifically biochemically binding to the ligand, said radiolabelled reactant emitting radiation energy capable of activating the fluorescer upon the binding of the reactant to the ligand, the radiolabelled reactant then being disposed in close enough proximity to the support particles to cause the radiation energy from the radiolabelled reactant to activate the fluorescer to produce light energy, whereas any radiolabelled reactant that does not bind to the
 - 15 ligand is generally too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and
 - c) measuring the light energy emitted by the fluorescer with the entire quantities of the support particles, bound and unbound radiolabelled reactant together in aqueous suspension.
2. A competitive reactant assay process, comprising:
 - 20 a) combining together in an aqueous medium:
 - a sample to be assayed containing an unknown amount of cold ligand reactant;
 - a known quantity of radiolabelled ligand reactant; and
 - a plurality of support particles having fluorescer integrated therewith capable of emitting photons when activated by the radiation energy emitted by the radiolabelled ligand reactant and having ligand
 - 25 attached to the outer surface thereof, said ligand capable of indiscriminately binding with both said cold and radiolabelled ligand reactant whereupon the binding of the ligand with the radiolabelled ligand reactant positions the radiolabelled ligand reactant close enough to the support particles to activate the fluorescer to emit photons through the aqueous medium, whereas the unbound radiolabelled ligand reactant is generally positioned too far away from the support particles to enable the radioactive energy
 - 30 emitted thereby to activate the fluorescer; and
 - b) measuring the photons emitted by the fluorescer with the entire quantities of cold ligand reactant containing sample, bound and unbound radiolabelled ligand reactant and support particles combined together in the aqueous medium.
3. A process as claimed in claim 1 or claim 2, wherein the fluorescer is insoluble in water.
- 35 4. A process as claimed in any one of claims 1 to 3, wherein the fluorescer is diphenyloxazole.
5. A process as claimed in any one of claims 1 to 4, further including impregnating or integrating the support particles with the fluorescer by:
 - a) placing the fluorescer in solution in a solvent that is miscible in water;
 - b) adding the support particles to the solution, said support particles being porous to the solution;
 - 40 c) removing the support particles from the solution; and
 - d) exposing the support particles to water to precipitate the fluorescer impregnated or integrated therein.
6. A test kit for the detection of a ligand reactant that biochemically and specifically binds to a ligand in an aqueous medium with all of the components of the test kit together in the aqueous medium, said test kit
 - 45 comprising:
 - a) support particles, preferably in a predetermined quantity, having a fluorescer incorporated therewith and the ligand attached thereto; and
 - b) a radiolabelled reactant, preferably in a predetermined quantity, having substantially the same specificity for the ligand as the ligand reactant; said support particles being such that when said kit is in use
 - 50 any of said radiolabelled reactant bound to the ligand is positioned close enough to the fluorescer to activate the fluorescer by the radioactive energy transmitted through the aqueous medium from the radioactive reactant at a level corresponding to the proportion of the radiolabelled reactant to the ligand reactant present in the aqueous medium but if not bound to the ligand is too far away from the fluorescer to result in other than background levels of fluorescer activation.
7. A test kit as claimed in claim 6, wherein the fluorescer is incorporated into said support particles by:
 - 55 a) placing the fluorescer in solution in a solvent, e.g. dimethyl sulfoxide, that is miscible in water;
 - b) adding said support particles to the solution, said support particles being porous to the solution;
 - c) removing said support particles from the solution; and
 - d) exposing said support particles to water to precipitate the fluorescer.
8. A test kit as claimed in claim 6 or claim 7, wherein said fluorescer includes diphenyloxazole.
- 60 9. A test kit as claimed in any one of claims 6 to 8, wherein said radiolabelled ligand reactant emits either beta rays or produces auger electrons.
10. A method of forming a test kit as defined in any one of claims 6 to 8 comprising packaging together said support particles and radiolabelled ligand reactant optionally together with instructions for the use of
 - 65 said kit in a process as claimed in any one of claims 1 to 5.

EP 0 154 734 B1**Patentansprüche**

1. Verfahren zur Erfassung eines radioaktiv markierten Reaktanten, dadurch gekennzeichnet, daß es die folgenden Schritte umfaßt:
 - 5 a) In eine wäßrige Suspension wird eine Vielzahl von Trägerteilchen gegeben, die mit einem Fluoreszor imprägniert sind und an die Liganden gebunden wurden;
 - b) In die Suspensionsflüssigkeit wird ein radioaktiv markierter Reaktant hinzugefügt, welcher fähig ist, sich spezifisch mit dem Liganden biochemisch zu verbinden, wobei der genannte radioaktiv markierte Reaktant eine Strahlungsenergie aussendet, die den Fluoreszor aktivieren kann, sofern der Reaktant an den Liganden gebunden ist, wobei der radioaktiv markierte Reaktant dann nahe genug bei den
 10 Trägerteilchen angeordnet ist, daß seine Strahlungsenergie den Fluoreszor zur Aussendung von Licht aktiviert, wogegen jener Teil des radioaktiv markierten Reaktanten, der nicht an Liganden gebunden ist, im allgemeinen zu weit entfernt von den Trägerteilchen ist, als daß die radioaktive Energie fähig wäre, den Fluoreszor zu aktivieren; und
 - 15 c) Die vom Fluoreszor ausgesandte Lichtenergie wird zusammen mit der gesamten Menge von Trägerteilchen sowie der gemeinsamen Menge des gebundenen und ungebundenen radioaktiv markierten Reaktanten in der wäßrigen Suspension gemessen.
2. Verfahren zur kompetitiven Bestimmung von Reaktanten, dadurch gekennzeichnet, daß es die folgenden Schritte umfaßt:
 - 20 a) In einem wäßrigen Medium werden zusammengefügt:
 - eine zu analysierende Probe, welche eine unbekannte Menge eines kalten, mit Liganden reagierenden Stoffes enthält;
 - eine bekannte Menge eines mit Liganden reagierenden radioaktiv markierten Stoffes;
 - eine Vielzahl von Trägerteilchen, in die ein Fluoreszor integriert wurde, der fähig ist, Photonen
 25 auszusenden, wenn er durch die von dem mit Liganden reagierenden, radioaktiv markierten Stoff ausgesandte Strahlungsenergie aktiviert wird, und an deren Oberfläche Liganden gebunden sind, welche Liganden unterschiedslos sowohl den kalten als auch den radioaktiv markierten mit Liganden reagierenden Stoff binden können, wodurch die Bindung des Liganden mit dem radioaktiv markierten, mit dem Liganden reagierenden Stoff diesen Stoff nahe genug an die Trägerteilchen heranbringt, daß der Fluoreszor zur
 30 Emission von Photonen durch das wäßrige Medium hindurch angeregt wird, wogegen der ungebundene Teil des radioaktiv markierten, mit Liganden reagierenden Stoffes im allgemeinen zu weit weg von den Trägerteilchen angeordnet ist, als daß seine radioaktive Energie den Fluoreszor anregen könnte; und
 - b) die vom Fluoreszor ausgesandten Photonen werden zusammen mit den gesamten Mengen sowohl der kalten mit Liganden reagierenden Stoff enthaltenden Probe als auch der in dem wäßrigen Medium
 35 befindlichen gebundenem und ungebundenem radioaktiv markierten mit Liganden reagierendem Stoff und der Trägerteilchen gemessen.
3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß der Fluoreszor in Wasser unlöslich ist.
4. Verfahren nach einem der Ansprüche 1 bis 3, dadurch gekennzeichnet, daß der Fluoreszor
 40 Diphenyloxazol ist.
5. Verfahren nach einem der Ansprüche 1 bis 4, dadurch gekennzeichnet, daß zum Imprägnieren der Trägerteilchen mit dem Fluoreszor oder zur Integration des Fluoreszors in die Trägerteilchen die folgenden Schritte durchgeführt werden:
 - a) der Fluoreszor wird in einem Lösungsmittel, das mit Wasser mischbar ist, gelöst;
 - 45 b) die Trägerteilchen werden zur Lösung hinzugefügt, wobei die Trägerteilchen für die Lösung porös sind;
 - c) die Trägerteilchen werden aus der Lösung entfernt; und
 - d) die Trägerteilchen werden in Wasser geschwemmt, um den in den Teilchen befindlichen Fluoreszor niederzuzuschlagen.
- 50 6. Testsatz für die Erfassung eines mit Liganden reagierenden Stoffes, der sich spezifisch mit einem Liganden in einem wäßrigen Medium biochemisch verbindet, wobei alle Bestandteile des Testsatzes sich in dem wäßrigen Medium befinden, dadurch gekennzeichnet, daß er folgendes umfaßt:
 - a) Trägerteilchen, vorzugsweise in einer vorgegebenen Menge, in die ein Fluoreszor eingebaut ist und an die ein Ligand gebunden ist; und
 - 55 b) ein radioaktiv markierter Reaktant, vorzugsweise in einer vorgegebenen Menge, der im wesentlichen dieselbe Spezifität für den Liganden besitzt wie der mit dem Liganden reagierende Stoff; wobei die Trägerteilchen so beschaffen sind, daß, wenn der Testsatz verwendet wird, der gesamte an Liganden gebundene Teil des radioaktiv markierten Reaktanten nahe genug am Fluoreszor angeordnet ist, daß der Fluoreszor durch die vom radioaktiven Reaktanten über das wäßrige Medium übertragene
 60 radioaktive Energie in einem Maße aktiviert wird, welches dem Verhältnis des radioaktiv markierten Reaktanten zum mit Liganden reagierenden Stoff in dem wäßrigen Medium entspricht, wogegen der Teil, der nicht an Liganden gebunden ist, zu weit vom Fluoreszor entfernt ist, als daß eine Anregung des Fluoreszors, die über den Untergrundpegel hinausgeht, bewirkt würde.
7. Testsatz nach Anspruch 6, dadurch gekennzeichnet, daß der Fluoreszor in besagte Trägerteilchen
 65 durch folgende Maßnahmen eingebaut wurde:

EP 0 154 734 B1

- a) der Fluoreszor wird in einem Lösungsmittel, z.B. Dimethylsulfoxid, das mit Wasser mischbar ist, gelöst;
- b) die genannten Trägerteilchen werden der Lösung hinzugefügt, wobei die Trägerteilchen für die Lösung porös sind;
- 5 c) die genannten Trägerteilchen werden aus der Lösung entfernt; und
- d) die genannten Trägerteilchen werden in Wasser geschwemmt, sodaß der Fluoreszor niedergeschlagen wird.
8. Testsatz nach Anspruch 6 oder 7, dadurch gekennzeichnet, daß der Fluoreszor Diphenyloxazol einschließt.
- 10 9. Testsatz nach einem der Ansprüche 6 bis 8, dadurch gekennzeichnet, daß der radioaktiv markierte, mit Liganden reagierende Reaktant entweder Beta-Strahlen aussendet oder Auger-Elektronen erzeugt.
10. Verfahren zur Herstellung eines Testsatzes nach einem der Ansprüche 6 bis 8, dadurch gekennzeichnet, daß die Trägerteilchen mit dem radioaktiv markierten, mit Liganden reagierenden Reaktanten zusammengepackt werden, wahlweise zusammen mit Anleitungen zur Verwendung des
- 15 Testsatzes in einem Verfahren nach einem der Ansprüche 1 bis 5.

Revendications

1. Procédé de détection d'un réactant radiomarqué, qui comprend:
- 20 a) l'introduction, dans une suspension aqueuse, d'une pluralité de particules de support imprégnées d'un agent fluorescent et sur lesquelles des ligands ont été attachés;
- b) l'addition, au liquide de la suspension, d'un réactant radiomarqué capable de se fixer biochimiquement de façon spécifique sur le ligand, le réactant radiomarqué émettant de l'énergie rayonnante capable d'activer l'agent fluorescent lors de la fixation du réactant sur le ligand, le réactant
- 25 radiomarqué étant ensuite disposé à proximité suffisamment étroite des particules de support pour amener l'énergie rayonnante de réactant radiomarqué à activer l'agent fluorescent pour produire de l'énergie lumineuse, tandis que tout réactant radiomarqué qui ne se fixe pas sur le ligand est généralement trop éloigné des particules de support pour permettre que l'énergie rayonnante active l'agent fluorescent; et
- 30 c) la mesure de l'énergie lumineuse émise par l'agent fluorescent avec les quantités totales des particules de support et de réactant radiomarqué fixé et non fixé conjointement dans la suspension aqueuse.
2. Procédé de dosage d'un réactant compétitif, qui comprend:
- a) la combinaison conjointe dans un milieu aqueux:
- 35 d'un échantillon à doser contenant une quantité inconnue d'un ligand réactant froid;
- d'une quantité connue de ligand réactant radiomarqué; et
- d'une pluralité de particules de support auxquelles est intégré un agent fluorescent capable d'émettre des photons lorsqu'il est activé par l'énergie rayonnante émise par le ligand réactant radiomarqué et portant un ligand attaché à leur surface extérieure, ce ligand étant capable de se fixer sans discrimination
- 40 sur le ligand réactant tant froid que radiomarqué, après quoi la fixation du ligand sur le ligand réactant radiomarqué positionne le ligand réactant radiomarqué suffisamment près des particules de support pour activer l'agent fluorescent pour qu'il émette des photons à travers le milieu aqueux, tandis que le ligand réactant radiomarqué non fixé est de façon générale positionné trop loin des particules de support pour permettre que l'énergie radioactive émise par lui active l'agent fluorescent; et
- 45 b) la mesure des photons émis par l'agent fluorescent avec les quantités totales d'échantillon contenant le ligand réactant froid, de ligand réactant radiomarqué fixé et non fixé et de particules de support conjointement dans le milieu aqueux.
3. Procédé suivant la revendication 1 ou 2, dans lequel l'agent fluorescent est insoluble dans l'eau.
4. Procédé suivant l'une quelconque des revendications 1 à 3, dans lequel l'agent fluorescent est le
- 50 diphenyloxazole.
5. Procédé suivant l'une quelconque des revendications 1 à 4, comprenant de surcroît l'imprégnation ou l'intégration des particules de support avec l'agent fluorescent
- a) en mettant l'agent fluorescent en solution dans un solvant qui est miscible à l'eau;
- b) en ajoutant les particules de support à la solution, ces particules de support étant poreuses pour la
- 55 solution;
- c) en retirant les particules de support de la solution; et
- d) en exposant les particules de support à de l'eau pour précipiter l'agent fluorescent qui y est imprégné ou intégré.
6. Trousse d'essai pour la détection d'un ligand réactant qui se fixe biochimiquement de façon
- 60 spécifique sur un ligand dans un milieu aqueux avec tous les composants de la trousse d'essai conjointement dans le milieu aqueux, la trousse d'essai comprenant:
- a) des particules de support, de préférence en une quantité déterminée au préalable, portant un agent fluorescent qui leur est incorporé et un ligand qui y est attaché, et
- b) un réactant radiomarqué, de préférence en une quantité déterminée au préalable, ayant
- 65 sensiblement la même spécificité pour le ligand que le ligand réactant; les particules de support étant telles

EP 0 154 734 B1

que lorsque la trousse est un usage, tout ce qui du réactant radiomarqué s'est fixé sur le ligand est positionné suffisamment près de l'agent fluorescent pour activer l'agent fluorescent par l'énergie radioactive transmise à travers le milieu aqueux à partir du réactant radioactif en une proportion correspondant au rapport du réactant radiomarqué au ligand réactant présent dans le milieu aqueux, mais ce qui n'est pas fixé sur le ligand est trop éloigné de l'agent fluorescent pour mener à autre chose qu'un activation aléatoire naturelle de l'agent fluorescent.

7. Trousse d'essai suivant la revendication 6, dans laquelle l'agent fluorescent est incorporé aux particules de support

a) en mettant l'agent fluorescent en solution dans un solvant, par exemple le diméthylsulfoxyde, qui est miscible à l'eau;

b) en ajoutant les particules de support à la solution, les particules de support étant poreuses pour la solution;

c) en retirant les particules de support de la solution; et

d) en exposant les particules de support à de l'eau pour précipiter l'agent fluorescent.

8. Trousse d'essai suivant la revendication 6 ou 7, dans laquelle l'agent fluorescent inclut du diphényloxazole.

9. Trousse d'essai suivant l'une quelconque des revendications 6 à 8, dans laquelle le ligand réactant radiomarqué émet des rayons bêta ou des électrons Auger.

10. Procédé pour former une trousse d'essai telle que définie dans l'une quelconque des revendications 6 à 8, qui comprend l'emballage des particules de support avec le ligand réactant radiomarqué; facultativement avec des instructions pour l'usage de la trousse dans un procédé suivant l'une quelconque des revendications 1 à 5.

EP 0 154 734 B1

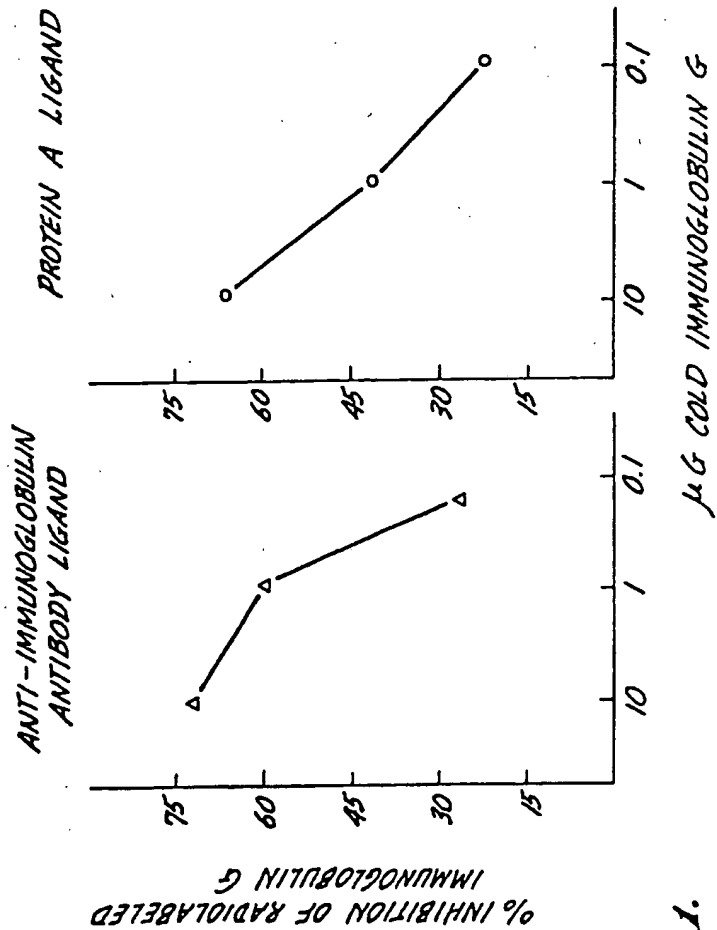
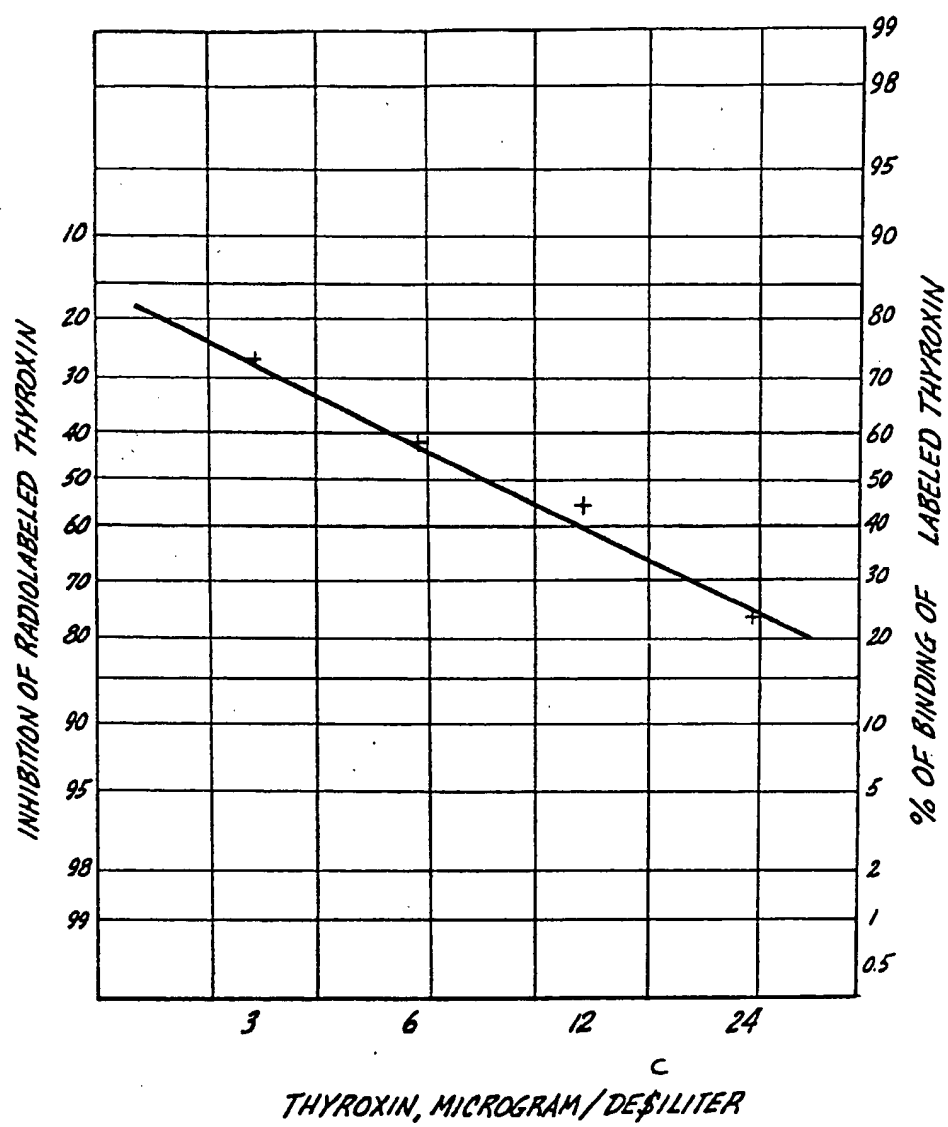


Fig. 1.

EP 0 154 734 B1

*Fig. 2.*